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Photosynthetic, morphological and biochemical biomarkers as tools to investigate copper oxide nanoparticle toxicity to a freshwater chlorophyceae☆

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ABSTRACT

Copper oxide nanoparticles (CuO NP) have been produced on a large scale due to their economically interesting thermophysical properties. This heightens the concern about risks they may pose on their release into the environment, possibly affecting non-target organisms. Microalga are important organisms in ecotoxicological studies as they are at the base of the aquatic food chain, but information about their biochemical and photosynthetic changes in response CuO NP are still scarce. We studied the effects of CuO NP in *Raphidocelis subcapitata* using morphological, photosynthetic and biochemical biomarkers. Our results showed that the NP affected microalgal population growth with 0.70 mg Cu L^{-1} $IC_{50-96\ h}$ (inhibition concentration). Based on predicted environmental concentrations of Cu NPs in aquatic environments, our results indicate potential risks of the NP to microalgae. Algal cell size, granularity and photosynthetic efficiencies were affected by the CuO NP at 0.97 and 11.74 mg Cu L^{-1}. Furthermore, lipid metabolism was affected mostly at the highest NP concentration, but at environmentally relevant values (0.012 and 0.065 mg Cu L^{-1}) the production of sterols (structural lipids) and triacylglycerols (reserve lipid) increased. Moreover, we found evidence of cell membrane impairment at the highest CuO NP concentration, and, as a photosynthetic response, the oxygen evolving complex was its main site of action. To the best of our knowledge, this is the first study to date to investigate microalgal lipid composition during CuO NP exposure, showing that it is a sensitive diagnostic tool. This research demonstrated that CuO NP may affect the physiology of *R. subcapitata*, and because they were observed in a primary producer, we foresee consequences to higher trophic levels in aquatic communities.

1. Introduction

Nanomaterials have received much attention in recent years because of their intrinsic properties, which include a high surface area to volume ratio and high reactivity (Bondarenko et al., 2013; Chang et al., 2012). Metallic nanoparticles (MNP) have been produced on a large scale for industrial and domestic applications (Aruoja et al., 2009; Chang et al., 2012), which results in the highest predicted environmental concentrations (PEC) compared to other

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types of nanoparticles (NP) (Wang et al., 2016).

Copper oxide nanoparticles (CuO NP) are MNP known to have high electric and thermal conductivity, as well as biocide effects due to their antibacterial and antifungal properties, and are widely used in industrial activities, such as in gas sensors, batteries, plastics, metal coating, semiconductors, electronic chips and, especially antifouling paints (Bondarenko et al., 2013; Gomes et al., 2012; Gunawan et al., 2011; Siddiqui et al., 2015). Negative effects of CuO NP released in aquatic environments can be better understood through the knowledge of their mobility, bioavailability and toxicity to organisms (Aruoja et al., 2009; Gomes et al., 2012; Siddiqui et al., 2015). Deleterious effects have already been reported in bacteria (Bondarenko et al., 2012; Heinlaan et al., 2008; Gunawan et al., 2011), algae (Aruoja et al., 2009; Joonas et al., 2019; Melegari et al., 2013), aquatic plant (Yue et al., 2018; Zhao et al., 2017), protozoan (Li et al., 2012), Cladocera (Griffit et al., 2008; Mansano et al., 2018; Thit et al., 2016), Oligochaeta (Amorim and Scott-Fordsmand, 2012; Gomes et al., 2012), Cnidarian (Siddiqui et al., 2015) and fish (Mansano et al., 2018; Song et al., 2015).

Among the most used organisms in ecotoxicological assessments, microalgae stand out as important model organism, and are widely used due to their sensitivity to many pollutants (Aruoja et al., 2009; Herlory et al., 2013; Joonas et al., 2019), their ecological relevance as primary producers, supporting all the other aquatic trophic levels (Melegari et al., 2013), as well as their fast growth and the fact that they are easy to maintain. It is known that the toxic effects of CuO NPs in aquatic organisms have the contribution of released Cu^{2+} ions (Aruoja et al., 2009; Heinlann et al., 2008; Wan et al., 2018). CuO NP toxicity can be partially attributed to oxidative stress caused by the generation of reactive oxygen species (ROS), regardless of whether this generation is due to Cu^{2+} dissolution from NPs or due to CuO NP properties (Fazelian et al., 2019; Mansano et al., 2018). However, in spite of the existing investigations, the toxicity mechanism and sites of action of CuO NP in algal cells are still not totally understood (Che et al., 2018; Fazelian et al., 2019).

Over the last decade several studies have evaluated possible aquatic contamination and toxic effects of CuO NP in microalgae (Aruoja et al., 2009; Joonas et al., 2019; Melegari et al., 2013; Wang et al., 2011). However, information about biochemical and photosynthetic changes in response to CuO NP effects are still scarce. Knowing that some NP can affect the photosynthetic apparatus of algae and plants, the pulse amplitude-modulated (PAM) fluorescence technique is a promising tool to evaluate where and how these NP interfere in the photosynthetic process (Joonas et al., 2019; Tighe-Neira et al., 2018).

Metal contamination may lead to changes in algal metabolic pathways, affecting the production of energy reserve compounds and lipid classes inside the cells (Chia et al., 2013; Liu et al., 2008; Rocha et al., 2016). It is highly important for ecotoxicological studies to detect and study these alterations as microalgae are primary producers and any impact on photosynthetic or biochemical levels may be transferred through the food chain, affecting other trophic levels, which can cause interference in the entire aquatic community of the contaminated environment.

Despite efforts to show the risks of CuO NP toxicity in aquatic environments, shown by the large number of studies regarding CuO NP effects on many different organisms (Bondarenko et al., 2012; Melegari et al., 2013; Zhao et al., 2017; Mansano et al., 2018; Song et al., 2015), currently the available risk assessment of nanoparticles is based on procedures originally established for conventional chemicals (Sørensen et al., 2018). Exposure assessments with model organisms using more robust and in-depth analyses are important for hazard identifications of these nanomaterials. According to Sørensen et al. (2018), hazard identification is one of the steps for establishing risk assessments. Therefore, by providing information about CuO NPs effects at photosynthetic and biochemical levels in microalgae, our study contributes by supplying knowledge regarding potential risks of CuO NP in aquatic systems.

To completely understand how and to what extent the CuO NP affects *R. subcapitata*, we evaluated different endpoints (population growth inhibition, cell size and granularity, chlorophyll *a* content, maximum and effective quantum yields, fluorescence quenching, total carbohydrates and lipids, and lipid class composition) at environmentally relevant (based on results of PEC estimated by Chio et al., 2012), and high CuO NP concentrations. Using these endpoints can improve ecotoxicity tests, providing more detailed information on the algal morphological, physiological and biochemical status (Alho et al., 2019). To the best of our knowledge this is the first study reporting CuO NP effects on a microalga assessed by these photosynthetic and biochemical biomarkers simultaneously, especially regarding information on lipid class composition.

2. Material and methods

2.1. Algal culture

Freshwater microalgae *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J. Kristiansen & O.M.Skulberg (Chlorophyceae) were obtained from the Department of Ecology and Evolutionary Biology, at the Federal University of São Carlos (São Carlos, SP, Brazil). Stock cultures were kept in L.C. Oligo culture medium (AFNOR, 1980) (Table S1, Supplementary material). The microalga was cultured at pH 7.0, under controlled conditions of light intensity (130 μmol photons m^{-2} s^{-1}) and temperature (25 ± 0.5 °C). The cultures were gently shaken manually 6 times a day (US EPA, 2012). The culture medium was sterilized through autoclaving for 20 min (at 121 °C and 1 atm above standard pressure). Sterile materials were used throughout the experiment to avoid culture contamination.

2.2. Flow cytometric analysis

Cell density was measured each 24 h by flow cytometer (FACSCalibur - Becton Dickinson, USA) equipped with a 15 mW Argon-ion laser (emission 488 nm). Formaldehyde at 1% final concentration fixed samples were flash-frozen in liquid nitrogen and stored at −20 °C until analysis. Sample storage time was always less than 1 month. Chlorophyll *a* fluorescence was detected as red fluorescence in a FL3 channel (660–700 nm). Cell count was performed according to Sarmento et al. (2008), using 6 μm fluorescent microspheres (Polysciences, Inc., Warrington, PA, USA.) as the internal standard. Microalgae were identified in side-scatter graphs (SSC–H) against red fluorescence (FL3-H). Data acquisition was done using CellQuest BD software and cytograms analysis using FlowJo V10 software. The extraction of mean values of FL3-H (chlorophyll *a* fluorescence), SSC-H (cell granularity) and FSC-H (cell size) of the microalgae and the bead population enabled us to calculate relative FL3-H ($FL3\text{-}H_{algae}/FL3\text{-}H_{beads}$), relative SSC-H ($SSC\text{-}H_{algae}/SSC\text{-}H_{beads}$) and relative FSC-H ($SSC\text{-}H_{algae}/SSCH_{beads}$), which were expressed in arbitrary units (Mansano et al., 2017).

2.3. Nanoparticle toxicity tests and ionic copper reference

A stock solution of CuO NP (CuO II, <50 nm, CAS number 1317-38-0, Sigma-Aldrich, USA) of 76.26 mg Cu L^{-1} was prepared in the culture medium and then sonicated for 30 min (DES 500, Unique

Brazil), 99% potency (Aruoja et al., 2009) in order to reduce the formation of aggregates. The test solutions were prepared through serial dilutions of the stock solution. The control treatment contained algae and the copper concentration already present in L.C. Oligo culture medium (0.004 mg Cu L^{-1}), with no extra copper added.

Considering that in some receiving waters, the PEC for Cu NP was 0.06 (95% confidence interval: 0.01–0.92 mg L^{-1}) (Chio et al., 2012), microalgae cells in exponential growth phase were exposed for 96 h to CuO NP at different concentrations: three low concentrations that are considered environmentally relevant according to the PEC, corresponding to 0.006, 0.012 and 0.065 mg Cu L^{-1}; and two high concentrations, 0.97 and 11.74 mg Cu L^{-1}. CuO NP concentrations were chosen based on preliminary test results: the lower ones within PEC values, the 0.97 mg Cu L^{-1} close to the PEC upper limit and the highest concentration (11.74 mg Cu L^{-1}) was previously determined to not kill the algal cells. Experiments began with 10^4 cells mL^{-1} initial cell density and 4 replicates per treatment. Continuous illumination was used throughout. The laboratory-controlled conditions were the same as described in Section 2.1. (Algal culture). Toxicity tests were performed in 500 mL polycarbonate Erlenmeyer flasks containing 250 mL of exposure medium.

An experiment with copper in its ionic form was performed and the concentration of 0.02 mg Cu L^{-1} was used as the ionic copper reference for some comparisons and inferences in the present research. For this ionic copper experiment, four concentrations (0.004, 0.008, 0.02 and 0.03 mg Cu L^{-1}) were used. A stock solution (31.36 mg Cu L^{-1}) from $CuCl_2$ ($CuCl_2.2H_2O$; Sigma, USA) was made and the four tested concentrations were further prepared by serial dilutions from the stock solution. The ionic copper reference concentration was chosen based on the information that the CuO NP that we used had about 2% dissolution in aqueous media (Tonietto et al., in preparation for submission). This means that 0.97 mg Cu L^{-1} CuO NP should release around 0.02 mg Cu L^{-1} to the culture medium. The complete set of results from the ionic copper experiment ($CuCl_2$) to which *R. subcapitata* was exposed to is provided as supplementary material (Fig. S1, S2 and S3; Table S2) and it followed the procedures adopted for the analyses in the CuO NPs experiment.

2.4. Nanoparticle characterization

Morphological characteristics of CuO NP were obtained through Atomic Force Microscopy (AFM) in the 11.74 mg Cu L^{-1} solution at room temperature using a FlexAFM (Nanosurf AG, Liestal, Switzerland) in intermittent contact mode (300 KHz) (Fig. S4, supplementary material), and showed that the shape of the NP was spherical. Measurements of the lateral dimension and height distribution were determined by image analysis software (AFM images Gwyddion). In AFM, the sample is scanned by a piezoelectric sensor used for studying the surface properties of materials (Binnig et al., 1986). Polydispersity index values (PdI), Zeta potential and nanoparticle hydrodynamic diameter determinations were performed through Dynamic Light Scattering (DLS). Nanoparticle characterizations were determined in the exposure medium and in ultrapure water at the concentrations of 0.97, 11.74 and 76.26 mg Cu L^{-1} (stock solution) of CuO NP, using the Malvern Spectrometer Nano ZS90, at 0 h and 96 h (Table S3, Supplementary material).

2.5. Metal determination

Total copper concentrations in the control, $CuCl_2$ reference and CuO NP treatments, as well as in the CuO NP and $CuCl_2$ stock solutions were measured by inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 7000 Series, Thermo Scientific) (Tables S4 and S5, supplementary material). The control only had the copper present in the culture medium LC Oligo, with no extra copper added. Samples were acidified to 1% HNO_3 and kept frozen (−20 °C) until being analyzed. The calibration curve used for copper determinations is reported in Fig. S5 (supplementary material). To increase the precision in our data, metal concentrations are reported as determined, not nominal concentrations. It is well known that nominal values do not necessarily represent the real exposure concentration. In our case, the difference was more than 20%.

2.6. Chlorophyll a determination

Chlorophyll *a* (Chl *a*) concentration was determined in 10 mL samples, in triplicate. Cultures were filtered through cellulose ester membranes (0.45 μm pore size), and the pigment was extracted using dimethylsulfoxide (DMSO), according to Shoaf and Lium (1976). The optical density was determined at 664 nm and 630 nm using a HACH DR500 spectrophotometer (HACH Company, Loveland, CO, USA), after a period of 45 min in the dark with periodic shaking. Chl *a* content was calculated based on the equation described in Jeffrey and Humphrey (1975).

2.7. Photosynthetic biomarkers

2.7.1. Fluorescence and quenching parameters

The Chl *a* fluorescence parameters were determined for each treatment using a pulse amplitude modulated fluorometer (PHYTO-PAM® Fluorometer Analyzer, Heinz Walz, Germany) with an optical drive ED-101US/MP. The analyzed parameters are described in Table S6 (supplementary material). Three mL samples were obtained from each treatment replicate and dark-adapted for 15 min to allow the complete oxidation of Photosystem II (PSII) reaction centers (RC). The photosynthetic parameters of dark-adapted cells were measured daily, while the photosynthetic parameters of light-adapted cells were measured at 96 h of CuO NP exposure, after 15 min dark-acclimation period. All the measurements were performed as described in Alho et al. (2019).

2.8. Dry weight

Dry weight was quantified gravimetrically on previously baked glass fiber filters (400 °C for 12 h), which were cooled to room temperature in a desiccator and weighed in an analytical balance (Sartorius MC21S, ± 1 μg, Bradford, MA, USA). After 96 h of exposure to CuO NP and $CuCl_2$, 60 mL of the algal cultures were filtered and the biomass dried at 60 °C for 72 h.

2.9. Biochemical biomarkers

The quantification of energy reserve compounds was based on carbohydrates and lipid classes. This was carried out for each replicate of control, CuO NP and $CuCl_2$ treatments after 96 h-exposure, totalizing four replicates per treatment.

2.9.1. Total carbohydrates

Total carbohydrates were determined according to the methodology described in Liu et al. (1973). The method is based on the phenol-sulfuric reaction and anhydrous dextrose (Mallinckrodt Chemicals, USA) as a standard for the calibration curve (Fig. S6, supplementary material). A spectrophotometer (HACH DR 5000; HACH Company, Loveland, CO, USA) was used for sample readings at 485 nm wavelength.

2.9.2. Total lipid and lipid classes

Total lipids and lipid class extraction and determination followed the methodology described in Parrish et al. (1999). A volume of 60 mL from each culture was filtered in previously baked (400 °C, 8 h) glass fiber filters (Macherey-Nagel, Germany) and stored in previously washed (methanol and chloroform) glass flasks. The storage period did not exceed 3 days at −20 °C until extraction. During the extraction process (repeated three times), samples were sonicated for 5 min (Unique Group, Indaiatuba, Brazil), followed by 2 min of centrifugation at 3000 rpm (Eppendorf 5702 R, Germany). After that, the samples were concentrated using a rotary evaporator (RV05 S25, IKA, Germany) to a final volume of 0.5 mL. An Iatroscan MK6 (Mitsubishi, Kagaku Iatron Inc., Tokyo, Japan), which consists of thin layer chromatography with flame ionization detection (TLC/FID), was used for lipid detection. This was based on a mixed lipid standard (Sigma-Aldrich, USA) calibration curve. Peak areas were recorded and processed by the PeakSimple software (version 4.444).

2.10. Statistical analysis

CuO NP inhibitory concentrations (IC) were calculated using cell density (cell mL^{-1}) measured daily, with a linear interpolation method using the ICPIN 2.0 software (US EPA, Duluth, Mn, USA). The Shapiro-Wilk test was performed to evaluate the normality of the data and the Levene median test was used for equal variance analysis. Data with normal distribution were submitted to One-Way ANOVA, followed by Dunnett's post-hoc tests. Data with non-normal distribution or unequal variance were analyzed using Kruskal-Wallis test and non-parametric Dunnett's post-hoc tests. The significant differences between treatments and control were considered for the $p < 0.05$ level. Statistical analyses were carried out using SigmaPlot version 11.0 software (Systat, 2008).

3. Results and discussion

3.1. Nanoparticle characterization

The data of CuO NP characterization in L.C. Oligo culture medium and in ultrapure water are reported in Table S3 (supplementary material). The results of Zeta-potential showed a trend to stability between 0 and 96 h at each concentration (high negative values). However, CuO NPs in the stock solution (76.26 mg Cu L^{-1}) showed a tendency to aggregate, leading to an NP size increase. It is known that zeta potential is affected by the ionic strength of the culture medium and the concentration of the particles (Lu and Gao, 2010; Melegari et al., 2013; Shang et al., 2014), where the NP was dispersed. The greater the value of NP surface charge (±30 mV), the greater the suspension stability due to repulsion of charges to overcome the natural tendency to the agglomeration of NP (Mahmoudi et al., 2011; Melegari et al., 2013).

In relation to the NP size, we found a tendency of formation of large aggregates, from 249 to 549 nm, as shown in Table S3. The aggregation over the experimental time may have occurred due to the constitution of the L.C. Oligo culture medium as the nutrients and ionic strength of culture medium can favor the NP interactions and aggregation (Bondarenko et al., 2013; Lu and Gao, 2010). In addition, the increase in CuO NP concentrations and exposure time may also be connected with the aggregation of NP. This is in accordance with other studies that showed NP aggregation (Amorim and Scott-Fordsmand, 2012; Bondarenko et al., 2013; Melegari et al., 2013; Mansano et al., 2018).

Fig. S7 (supplementary material) shows the size distribution based on the diameter of CuO NP that was measured by AFM and DLS. It indicates larger aggregates in the samples measured by DLS. Once more, it should be mentioned here that NP concentration can be an important factor in aggregation and for this analysis, 11.74 mg Cu L^{-1} CuO NP was used.

3.2. Population growth inhibition and flow cytometric analysis

The exposure to environmentally relevant concentrations of 0.012 and 0.065 mg Cu L^{-1} of CuO NP led to a stimulus of algal growth. This is not unusual and may be referred to as the *hormesis effect* (Rand, 1995). Echeveste et al. (2017) and Wan et al. (2018) obtained higher growth of *Chlorolobion braunii* and *Chlorella* sp. Exposed to low concentrations of Cu and CuO NP, respectively. According to Calabrese and Mattson (2017), *hormesis* can be defined as adaptive responses of cells and organisms, which can be characterized as a stimulation of cellular functions/endpoints to an imposed or intrinsically generated challenge through which the system improves its ability and/or tolerance to deal with more serious challenges. Thus, not only microalgae, but the biota in general can be stimulated under exposure to low concentrations of chemicals. In fact, several publications show that this can also be detected in zooplankton (De Schamphelaere and Janssen, 2004; Gusso-Choueri et al., 2012; Sánches-Ortíz et al., 2010; Sarma et al., 2008).

The cell division ability in *R. subcapitata* decreased just at the higher end of CuO NP concentrations, e.g. 0.97 mg Cu L^{-1} with 64% inhibition and 11.74 mg Cu L^{-1} with 95%. This was also detected in the ionic copper reference (0.02 mg Cu L^{-1}) (Fig. 1A). This effect had its repercussion in IC values, which are reported in Table 1. These results confirm that after 48 h exposure, the decrease in cell division capacity was already underway and did not change thereafter. While 0.70 mg Cu L^{-1} was necessary to inhibit 50% of the population growth, half that would inhibit 25%, and 10% would be inhibited in even less CuO NP concentration. These results may have important ecological consequences if we consider that, except for the presence of CuO NPs, the microalga was grown under ideal conditions with no other stress taking place. This is a rare situation in nature, where more than one stressor may often be present and synergistic effects may occur. We emphasize that the IC values are within the confidence interval of PEC for Cu NP (0.010–0.92 mg L^{-1}) (Chio et al., 2012), showing that CuO NP represents risks to *R. subcapitata* in aquatic systems. The IC_{50} obtained in the present research is in accordance with that reported in Aruoja et al. (2009) when exposing the same species to CuO NP. The IC_{50} they obtained was 0.71 mg Cu L^{-1}.

Considering that the CuO NP used in this research dissolves at a proportion of 2% into inorganic media, the IC_{50} of 0.70 mg Cu L^{-1} corresponds to 0.014 mg Cu L^{-1}, almost exactly the dissolved copper concentration that inhibited 50% of the population density in the ionic copper experiment (Table S2, supplementary material). This enables us to correlate the toxicity of CuO NP with the release of copper ions and confirms its contribution to the toxic effects observed. These data agree with what was reported in the literature, i.e. that some nanoparticles are generally less toxic than the ionic form of its constituent material, and that the release of such material into the surroundings is an important aspect of the observed toxicity, particularly in relation to cell division and growth rates (Aruoja et al., 2009; Franklin et al., 2007; Wan et al., 2018).

The flow cytometry analysis showed that exposure to CuO NP induced morphological alterations that can be visualized through cell size (FSC–H) and granularity (SSC–H) variations in 96 h exposure. Cell size increased in ionic copper exposed algae, and in both 0.97 and 11.74 mg Cu L^{-1} (Fig. 1B), but granularity was higher just at the highest CuO NP concentration. No effect of ionic copper

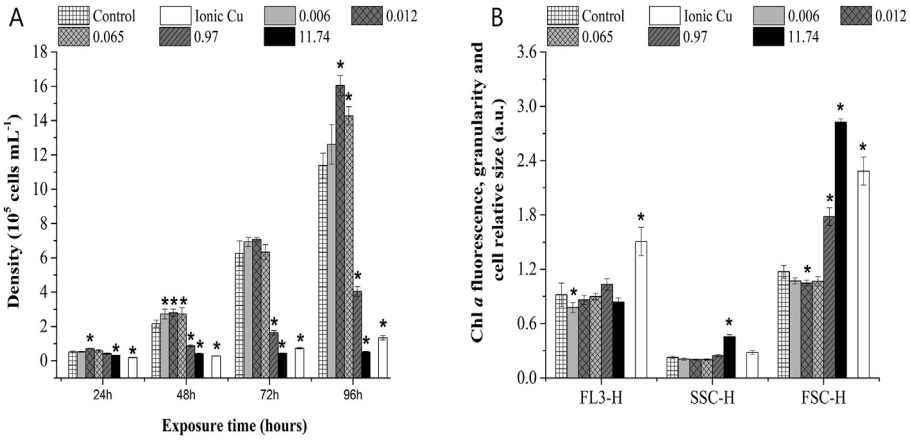

Fig. 1. (A) Cell density of *Raphidocelis subcapitata* during the 96 h of exposure to CuO nanoparticles, and (B) measurements of chlorophyll *a* fluorescence (relative FL3-H), granularity (relative SSC-H) and cell size (relative FSC-H) after 96 h of treatment. Concentrations are expressed in mg Cu L^{-1}. Ionic Cu represents the Ionic copper reference (0.02 mg Cu L^{-1} $CuCl_2$). Asterisks (*) represent the significant difference ($p < 0.05$) of treatments compared to the control group.

Table 1
Inhibitory concentration (IC) values for *Raphidocelis subcapitata* after exposure to copper oxide nanoparticles (CuO NP, in mg Cu L^{-1}) for 24, 48, 72 and 96 h. IC_{10}, IC_{25} and IC_{50} values represent the concentrations that inhibited 10%, 25% and 50%, respectively, of the algal growth (cells mL^{-1}). Results correspond to median and 95% confidence intervals. NC = not calculated.

Time of exposure	IC_{50}	IC_{25}	IC_{10}
24 h	NC	NC	NC
48 h	0.73 (0.69–0.76)	0.40 (0.36–0.41)	0.19 (0.14–0.20)
72 h	0.64 (0.60–0.66)	0.32 (0.25–0.36)	0.13 (0.05–0.18)
96 h	0.70 (0.69–0.72)	0.38 (0.37–0.39)	0.19 (0.19–0.19)

in granularity was observed. The higher cell granularity is in accordance with the results obtained by Melegari et al. (2013) who reported higher granularity in *Chlamydomonas reinhardtii* exposed to CuO NP concentrations. The authors were able to detect proportionality between CuO NP increase (from 0.1 to 1000 mg L^{-1}) and cell granularity. Several studies indicate that SSC intensity may be proportional to NP concentration inside the cells (Oukarroum et al., 2017; Park et al., 2017; Saison et al., 2010). The increase in cell size is a common feature in copper exposed cells and has been documented in the literature (Echeveste et al., 2017; Rocha et al., 2016; Silva et al., 2018). According to Lim et al. (2006), high Cu concentrations may damage cell membrane and decrease intracellular K^+ levels, which may facilitate Cu internalization. The excess intracellular Cu can affect enzymes and reduce reproduction processes by impairing cell division. Echeveste et al. (2017), Rocha et al. (2016) and Silva et al. (2018) suggested that cell size increase can be a response of algal machinery to reduce the surface to volume ratio, helping the cell survive in a stressing condition.

3.3. Chlorophyll a content and photosynthetic evaluation

The content of Chl *a* in *R. subcapitata* exposed to the highest tested CuO NP concentration (11.74 mg Cu L^{-1}) increased compared to the control, which happened after 96 h exposure (Fig. 2A). According to Silva et al. (2018) it can be an adaptive strategy to maximize light harvesting. This is supported by the fact that in the present study, it was just in the highest CuO NP concentration that a reduction of the effective photosynthetic quantum yield (Fig. 2D) was detected. Despite the similarity of Chl *a* values in 0.97 mg Cu L^{-1} and the control, Chl *a* in algal cells exposed to ionic copper was lower than the respective control. The highest CuO NP concentration also affected F_0/F_V (Fig. 2B), increasing it by 90.4% at 11.74 mg Cu L^{-1} in 48 h exposure compared to the control. This indicates that the water photo-oxidation process was impaired and suggests that the OEC of PSII was an action site of CuO NP. This was also observed for other metals (Cd, Cu, Ur and Zn) (Herlory et al., 2013; Juneau and Popovic, 1999; Juneau et al., 2002; Mallick and Mohn, 2003). Besides that, the alterations in the F_0/F_V values in the ionic copper and CuO NP concentrations of 0.97 and 11.74 mg Cu L^{-1}, during the 96 h of exposure, were followed by a reduction in the maximum quantum yield (ϕ_M) at these same concentrations (Fig. 2C). The lower ϕ_M can be related to a reduction in electron transport between algae photosystems, which results in a limitation of Q_A re-oxidation (Mallick and Mohn, 2003). Based on our results, this probably occurred due to the nanoparticle impacts on the OEC, which caused a reduction in the release of electrons from the photolysis of water molecules.

No changes in ϕ_M and ϕ'_M were observed in environmentally relevant CuO NP concentrations, but at the highest (Fig. 2D). This may indicate an impairment of electron transport between the PSII and PSI of the microalgae due to the effects of OEC and the water oxidation process (Alho et al., 2019; Juneau et al., 2002; Mallick and Mohn, 2003). The fluorescence parameters related to photosynthesis obtained in this research suggest that at 11.74 mg Cu L^{-1}, representing the highest CuO NP concentration, the inhibitory effect in PSII activity is located at the donor side of the reaction centers (RC). This rationale comes from the known fact that electron flow inhibition on the donor side of RC of PSII causes a reduction in Chl *a* fluorescence and that inhibition produced on the acceptor side of the RC of PSII would have induced an increase in Chl *a* fluorescence (Cid et al., 1995), which was not the case in our study. As a consequence of damaged OEC, the capacity of PSII to supply electrons to the RC is reduced in light, causing overproduction of ROS in chloroplast, as a result of singlet oxygen produced due to snatched electrons from O_2 by the RC (Che et al., 2018).

Optimal use of photochemical energy in carbon metabolism is characterized by qP values close to 1 (Lombardi and Maldonado, 2011) as this parameter represents the proportion of excitation energy captured by the RC of PSII that is used for electron transport (Genty et al., 1989; Herlory et al., 2013; Juneau et al., 2002). Based on the high qP values (Fig. 2D) found in the ionic copper and in all CuO NP concentrations, the algae were able to invest in photochemistry even at 11.74 mg Cu L^{-1}, but this did not occur. This rationale is supported by reduced values of ϕ_M and ϕ'_M, and suggests some damage to the electron transport mechanism. The non-

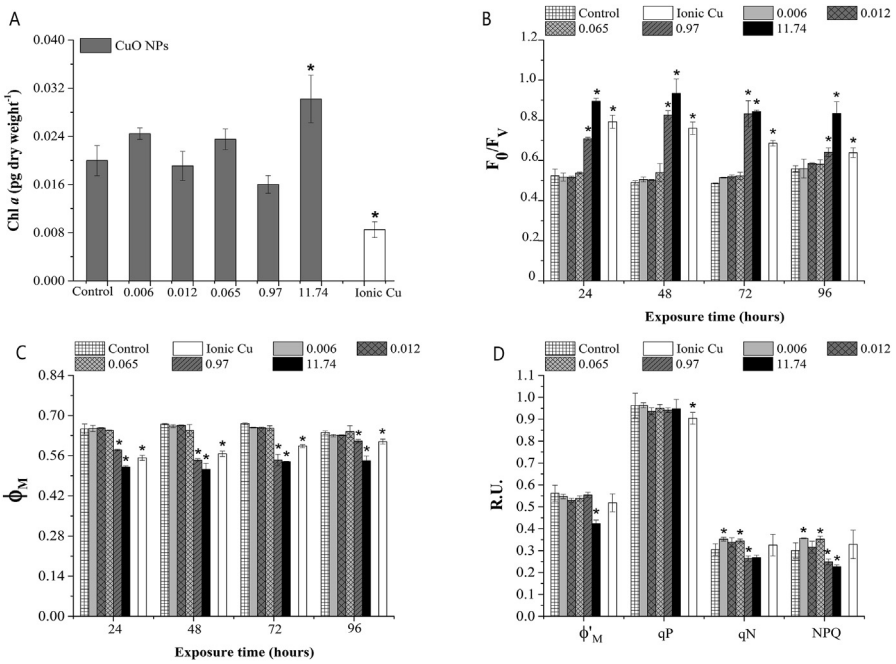

Fig. 2. Chlorophyll *a* (A), efficiency of oxygen evolving complex (F_0/F_V) (B) and maximum quantum yield (ϕ_M) (C) during exposure time; values of effective quantum yield (ϕ'_M) and quenching parameters (photochemical quenching – qP and non-photochemical quenchings - qN and NPQ) (D) of *Raphidocelis subcapitata* after 96 h of exposure to CuO nanoparticles (mg Cu L^{-1}). Ionic Cu represents the Ionic copper reference (0.02 mg Cu L^{-1} $CuCl_2$). Asterisks (*) represent a significant difference ($p < 0.05$) of treatments compared to the control group.

photochemical quenchings qN and NPQ that increased ~15% in cells exposed to the environmentally relevant concentrations of CuO NP (0.006 and 0.065 mg Cu L^{-1}) can indicate activation of photoprotection mechanisms (Herlory et al., 2013). However, at the highest concentration of CuO NP, the decrease of qN and NPQ indicates damage to the photosynthetic machinery and cells unable to activate photoprotection mechanisms.

3.4. Biochemical composition

This research showed increased intracellular carbohydrates in cells exposed to the highest CuO NP concentration (11.74 mgL^{-1} Cu), as shown in Fig. 3A. According to the literature, this can be a defense mechanism either to decrease copper bioavailability (Silva et al., 2018) or a protection to the cell wall, supporting its integrity (Markou et al., 2012). According to Markou et al. (2012), as structural and storage compounds, carbohydrates provide energy to cells to maintain metabolic processes and maintain cell wall integrity. Since CuO NP is a metallic nanoparticle, higher carbohydrate content in *R. subcapitata* exposed to the highest concentration (Fig. 3A) may be a defense mechanism, either to decrease bioavailability of the metal or to protect the cell wall. Due to the high growth inhibition caused by exposure to the ionic copper, there was no sufficient biomass to quantify carbohydrates and, therefore, no comparison with CuO NP of 0.97 mg Cu L^{-1} could be made.

Total lipid content in *R. subcapitata* exposed to 0.97 and 11.74 mgL^{-1} Cu as CuO NP were higher than the control (Fig. 3A), indicating that lipid metabolism was affected. The ionic copper reference in which *R. subcapitata* was exposed to 0.02 mg Cu L^{-1} as $CuCl_2$, showed total lipids higher than 0.97 mg Cu L^{-1} as CuO NP. Even though we did not measure copper dissolution from the NPs, if we consider its 2% dissolution, it would result in 0.02 mgL^{-1} Cu ions concentration in culture medium. It is known that lipids can act as an electron sink, combating damage caused by oxidative stress due to ROS formation. Chia et al. (2015) and Hu et al. (2008) proposed that higher lipid production favors the maintenance of microalgae redox homeostasis. In fact, this has been confirmed by Alho et al. (2019) who showed a decrease in ROS generation in microalgae under increased lipid production as a response to metal stress.

Our results demonstrated differences in the alteration of lipid class composition in response to the concentration of CuO NP (Figs. 3B and 4). Some classes were already altered at the environmentally relevant concentrations, and the effects on acetone-mobile polar lipids (AMPL), aliphatic alcohol (ALC) and free sterol (ST) were detected at concentrations (0.012 and 0.065 mg Cu L^{-1}) that did not lead to changes in other parameters, suggesting that lipid class compositions are good tools to investigate CuO NP effects in Chlorophyceae.

In relation to lipid class composition (Fig. 3B), our results showed that ionic copper reference differed from its equivalent in the form of NP (0.97 mgL^{-1} Cu as CuO NP). In the ionic form, less PL and more ST and AMPL were present, while in cells exposed to 0.97 mg Cu L^{-1} of CuO NP, we obtained 60% more triacylglycerols (TAG) and higher ST content, and reduced AMPL values. The concentrations of 0.012 and 0.065 mg Cu L^{-1} led to an increase in ST contents in about 6 and 5-fold, respectively, compared to the control group. AMPL increased at 0.012 mg Cu L^{-1} and ALC values reduced by approximately 83% at 0.065 mg Cu L^{-1}. Most alterations occurred at the highest concentration, with an increase in aliphatic hydrocarbons (HC) (142%), wax esters (WE) (77.5%) and free fatty acids (FFA) (241%), while the values of AMPL and phospholipids (PL) were reduced compared to the control. These reduced PL values suggest that the membrane may have been damaged. Many studies have shown that CuO NP induced oxidative stress in various organisms (Fazelian et al., 2019; Mansano et al., 2018; Melegari et al., 2013), which is usually accompanied by damage in membrane lipids as a consequence of the ROS harmful effects (Che et al., 2018; Fazelian et al., 2019). The damaged membrane may facilitate the

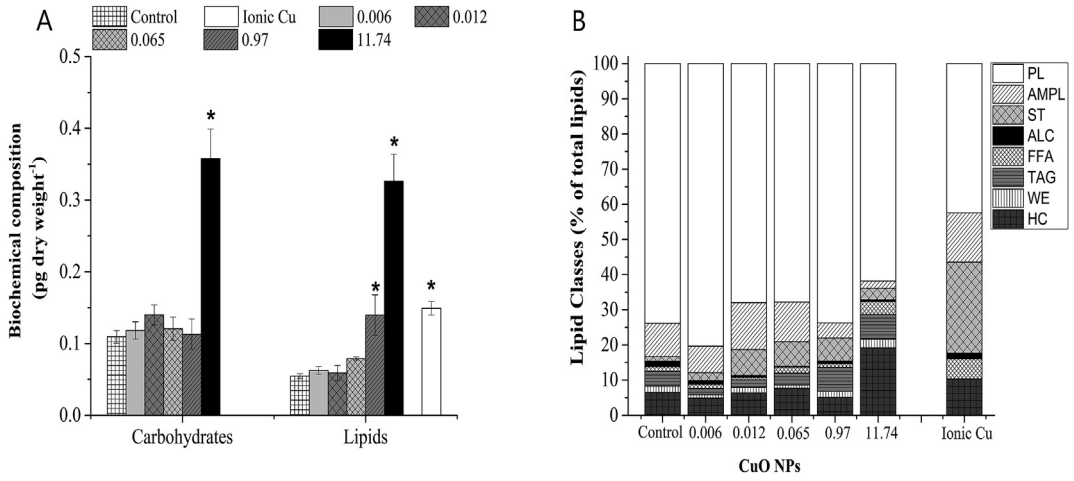

Fig. 3. Measurements of total carbohydrates and total lipids (A); lipid class composition in percentage of total lipids (B) of *Raphidocelis subcapitata* after 96 h exposure to CuO NP. Ionic Cu represents the Ionic copper reference (0.02 mg Cu L^{-1} $CuCl_2$). Lipid classes are HC (aliphatic hydrocarbons), WE (wax esters), TAG (triglycerides), FFA (free fatty acids), ALC (aliphatic alcohol), ST (free sterol), AMPL (acetone-mobile polar lipids) and PL (phospholipids). Concentrations are expressed in mg L^{-1} total copper. Asterisks (*) represent the significant difference ($p < 0.05$) of treatments compared to the control group.

internalization of CuO NPs into the cell, which may explain the increased value of SSC-H (granularity) at this highest concentration. However, measurements of CuO NP internalization by the algal cells must be performed to confirm this hypothesis.

The lipid classes HC, FFA, ALC and ST can generally be found in quantities lower than 10% in algal cells, even under stress conditions (Gushina and Harwood, 2006; Lombardi and Wangersky, 1991), but their proportions can be affected by metal stress, such as those found by Chia et al. (2013) when exposing *C. vulgaris* to Cd.

Considering the results of lipid classes per unit biomass (Fig. 4), the most altered classes by the CuO NP were found at the highest concentration, with an increase in the ST level, FFA and TAG content compared to the amount found in the control cells. At 0.97 mg Cu L^{-1}, the ST levels increased by about 13.7-fold and PL increase was of 2.3-fold. Furthermore, we found altered lipid class contents at the environmentally relevant concentrations (reduced ALC content and increased ST levels 0.012 mg Cu L^{-1} and an increase in ST and AMPL levels at 0.065 mg Cu L^{-1}). In general, cells exposed to ionic copper usually had higher lipids than those exposed to 0.97 mgL^{-1} of NPs. Based on these results, it can be observed that the effects of CuO NP on PL and ST were remarkable. In the case of ST, even environmentally relevant CuO NP concentrations lead to an increase in this lipid class. In addition, as CuO NP increased, so did ST, and under exposure to ionic copper ~3 times more ST was obtained compared to the highest sterol content under CuO NP. Phospholipids were significantly higher than the control in 0.97 and 11.74 mgL^{-1} CuO NP and ionic copper (0.02 mgL^{-1} Cu). The ionic copper also led to increased ALC and AMPL content.

Although PL was not the most altered lipid class, it was the most representative one, with ~60% in control and CuO NP concentrations. This indicates an investment in the algal cells to maintain membrane integrity, which is reinforced by the gradual increase of ST content with the increase in NP concentrations (Fig. 4B). An implication of this finding is that both protection and reinforcement of cell membrane were processes in which the microalga invested to deal with the adverse effects brought about by CuO NP. PL and ST are known to be important lipid classes as structural components in cell membranes, regulating their permeability and fluidity (Alho et al., 2019; Rocha et al., 2016).

The increased TAG level at the highest NP concentration may be related to a decrease in the transcription of lipase genes, as observed by Yang et al. (2013) in diatoms exposed to nitrogen limitation, which results in a lower TAG degradation. This can occur when the algal cell division is impaired, as we found in our study, and there is no further requirement for synthesis of new membrane components (Sharma et al., 2012), suggesting that TAG accumulation may act as a defense mechanism at these conditions. The increase in TAG content under metal stress was also reported by Alho et al. (2019) and Chia et al. (2015).

The responses of *R. subcapitata* to the different levels of CuO NP concentrations, especially at 0.97 and 11.74 mg Cu L^{-1}, indicated that different concentrations will trigger different metabolic responses. These biochemical alterations may pose as risks for aquatic food webs due to changes in microalgae nutritional values, affecting the quantity and/or quality of transferred energy to upper trophic levels.

The findings of this study suggest that the major negative effect of CuO NPs was in the photosynthetic apparatus. Cells exposed to 0.97 and 11.74 mg Cu L^{-1} had the oxygen-evolving complex as the main site of action of CuO NP, and inhibition of photosynthetic electron transport was an important toxicity response that impaired photosynthesis. Therefore, population growth reduction can be a consequence of decreased photosynthesis, as also shown by Che et al. (2018). At the same time, several biochemical alterations were manifested in the cells. They can be considered an algal response as an attempt to cope with the stress caused by the CuO NP.

Comparing the ionic reference with the CuO NP, in general *R. subcapitata* was more vulnerable to the ionic form of copper, $CuCl_2$, than to CuO NP. This is confirmed by the results of copper salt exposure on growth, photosynthesis and biochemical composition (supplementary material - Fig. S2, S2 and S3, and Table S2). In spite of the higher sensitivity to ionic copper, both $CuCl_2$ and CuO NP share a number of key features in algal response. They share the same damage site of action, which can be related to the substitution of M^{2+} on the water photo – oxidation apparatus by the Cu^{2+} ions, as previously suggested by Juneau and Popovic (1999).

In general, *R. subcapitata* showed an adaptation trend over the exposure time to the NP, especially at the highest concentrations, as suggested by the results of ϕ_M and F_0/F_V. The algae showed a capacity to cope with the CuO NP impacts at physiological and biochemical levels at the low and high concentrations potentially found in contaminated environments (0.006–0.97 mg Cu L^{-1}). Even at the highest concentration tested (11.74 mg Cu L^{-1}), the

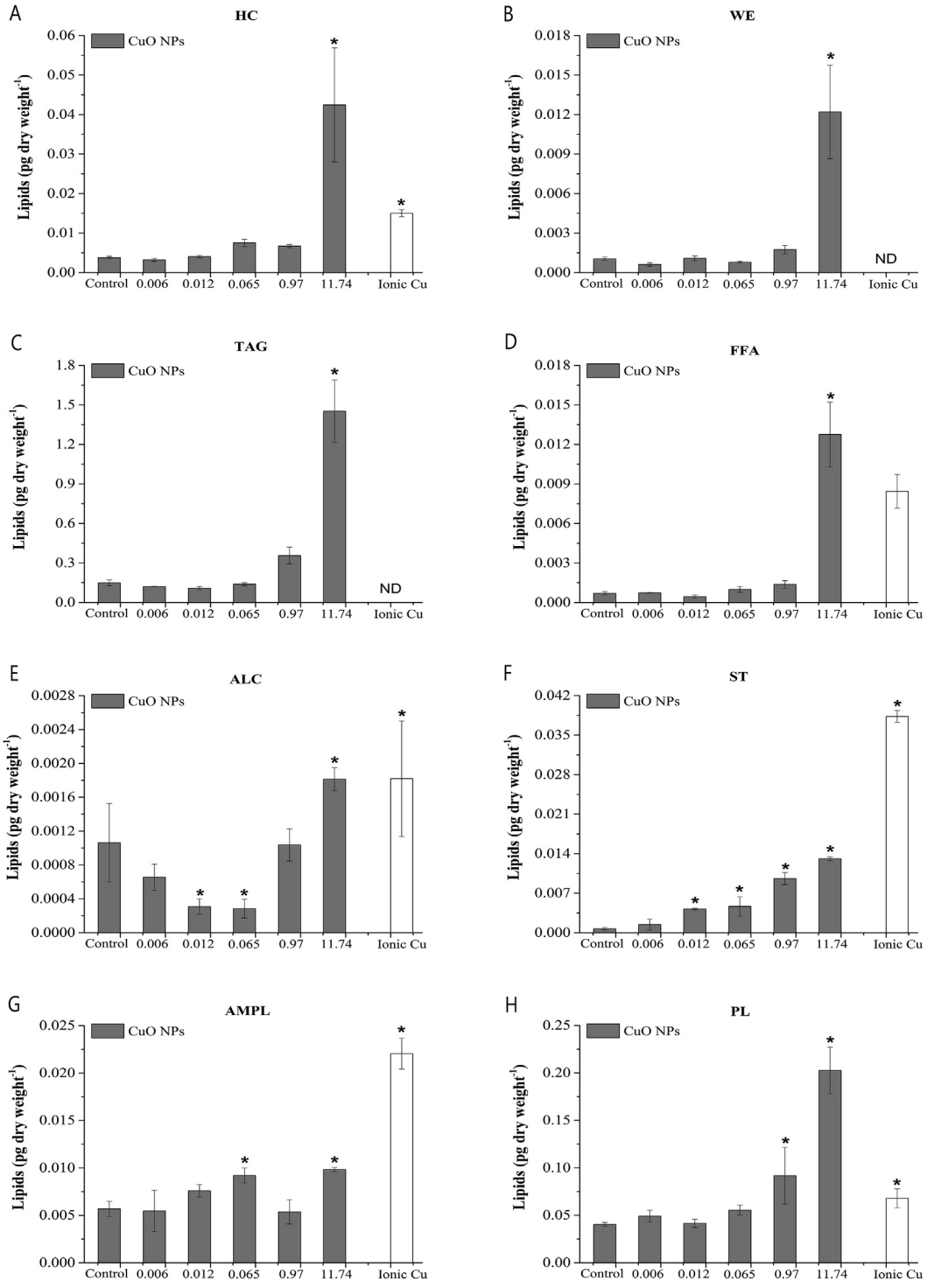

Fig. 4. Individual lipid class composition (pg cell^{-1}) of *Raphidocelis subcapitata* after 96 h of exposure to CuO nanoparticles (mg Cu L^{-1}). Ionic Cu represents the Ionic copper reference (0.02 mg Cu L^{-1} $CuCl_2$). Lipid classes are HC (A - aliphatic hydrocarbons), WE (B - wax esters), TAG (C - triacylglycerols), FFA (D - free fatty acids), ALC (E – aliphatic alcohol), ST (F - free sterol), AMPL (G - acetone-mobile polar lipids) and PL (H - phospholipids). Asterisks (*) represent the significant difference ($p < 0.05$) of treatments compared to the control group.

microalga used biochemical pathways to produce more energy reserve compounds and structural lipids, probably in an attempt to reduce CuO NP negative impacts on photosynthetic processes. This hypothesis is supported by the results of ϕ_M and F_0/F_V, which indicated a reduction in the impairment of photosynthetic apparatus throughout the exposure time when compared to the control. The changes in cell size and in some lipid classes were detected in concentrations as low as 0.012 and 0.065 mg Cu L^{-}1, which are environmentally relevant. Consequently, microalgae nutritional values may be altered and reach other trophic levels in aquatic environments, indicating these biomarkers as great tools to assess CuO NP toxicity to microalgae.

4. Conclusion

Our results provide new information regarding the risks of CuO NP in aquatic environment exploring responses at physiological and biochemical levels in microalgae, and can contribute to improvements in environmental risk assessment. Exposure to CuO NP, especially at high concentrations (0.97 mg L^{-1} and above), induced physiological, morphological and biochemical changes in *R. subcapitata* cells. An increase in cell size indicated impairments of the division process. Because the water-photolysis process provides electrons to the acyclic photosynthetic electron transport chain, we can deduce that the damage in the oxygen-evolving complex revealed by increased values of F_0/F_V led to a reduction in algal photosynthetic efficiency and reduced ϕ_M values. This demonstrates that the water splitting apparatus can be the main site of action of the CuO NPs. The results of IC_{50} demonstrated that CuO NP represent risks to some Chlorophyceae species, based on predicted environmental concentrations for Cu NPs in aquatic systems. We showed that intracellular lipid classes were affected in response to the different CuO NP levels, mostly ST, TAG and FFA at the highest CuO NP concentration. We suggest that the accumulation of structural lipids was probably an algal response to higher CuO NP concentrations in an attempt to keep cell membrane integrity through changing its fluidity and thickness, and reduce the damage caused by the NP. In general, the ionic copper results indicate that the release of Cu^{2+} ions was probably the origin of CuO NP toxicity as they shared similar toxicity targets and responses in the microalgae.

Author contributions

This manuscript describes original work and is not under consideration by any other journal. All authors assume that they have read the final version of the manuscript and agreed with it. The contribution of each author of this work is described below:

LOGA: co-developed the experimental design, carried out experimental tests and collected the data; performed statistical analysis; analyzed and interpreted the data and wrote the paper.

JPS: co-developed the experimental design; performed the characterization of CuO nanoparticles; analyzed and interpreted the data and reviewed the paper.

GSR: co-developed the experimental design; analyzed and interpreted the data and reviewed the paper.

ASM: performed the characterization of the CuO nanoparticles and the quantification of actual copper concentrations in ICP-OES (inductively coupled plasma optical emission spectrometry); analyzed and interpreted the data and reviewed the paper.

ATL: co-developed the experimental design; analyzed and interpreted the data and reviewed the paper.

HS: analyzed and interpreted the data and reviewed the paper. This author is also one of the sponsors, responsible for obtaining financial grant that supported this study (FAPESP, 2014/14,139−3).

MGGM: co-developed the experimental design; analyzed and interpreted the data and reviewed the paper. This author is also one of the sponsors, responsible for obtaining financial grant that supported this study (FAPESP, 2016/00753−7).

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114856.

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